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## Granulocyte colony-stimulating factor (G-CSF) depresses angiogenesis *in vivo* and *in vitro*: implications for sourcing cells for vascular regeneration therapy

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### Summary

**Background**—The most common source of hematopoietic progenitor cells (HPCs) for hematopoietic reconstitution comprises granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells (PBSCs). It has been proposed that endothelial progenitor cells (EPCs) share precursors with HPCs, and that EPC release may accompany HPC mobilization to the circulation following G-CSF administration.

**Objective**—To investigate EPC activity following HPC mobilization, and the direct effects of exogenous G-CSF administration on human umbilical vein endothelial cells (HUVECs) and endothelial outgrowth cells (EOCs), using *in vitro* and *in vivo* correlates of angiogenesis.

**Patients/Methods**—Heparinized venous blood samples were collected from healthy volunteers and from cord blood at parturition. G-CSF-mobilized samples were collected before administration, at apheresis harvest, and at follow-up. PBSCs were phenotyped by flow cytometry, and cultured in standard colony-forming unit (CFU)-EPC and EOC assays. The effect of exogenous G-CSF was investigated by addition of it to HUVECs and EOCs in standard tubule formation and aortic ring assays, and in an *in vivo* sponge implantation model.

**Results**—Our data show that G-CSF mobilization of PBSCs produces a profound, reversible depression of circulating CFU-EPCs. Furthermore, G-CSF administration did not mobilize CD34+CD133– cells, which include precursors of EOCs. No EOCs were cultured from any mobilized PBSCs studied. Exogenous G-CSF inhibited CFU-EPC generation, HUVEC and EOC tubule formation, microvessel outgrowth, and implanted sponge vascularization in mice.

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### Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

**Conclusions**—G-CSF administration depresses both endothelial cell angiogenesis and monocyte proangiogenic activity, and we suggest that any angiogenic benefit observed following implantation of cells mobilized by G-CSF may come only from a paracrine effect from HPCs.

### Keywords

angiogenesis; endothelial progenitor cells; granulocyte colony-stimulating factor; stem cell therapy

### Introduction

The recent discovery that endothelial progenitor cells (EPCs), which play a role in *de novo* vascularization (vasculogenesis), circulate in adult blood and reside in the bone marrow [1,2] prompted a range of studies based on localized implantation of autologous cells, aimed at vascularizing ischemic tissue, particularly in myocardial and critical limb ischemias. EPCs have not yet been definitively characterized, but have been linked with hematopoietic progenitor cells (HPCs). EPCs and HPCs share a common ancestor, the hemangioblast, in the developing fetus that may be retained in adult life [3]. HPCs derived from bone marrow [bone marrow stem cells (BMSCs)] or peripheral blood (PB) [peripheral blood stem cells (PBSCs)] following granulocyte colony-stimulating factor (G-CSF) administration have been utilized as sources of EPCs for regenerative vascularization. A recent meta-analysis showed that BMSC treatment generally improves short-term measurements of cardiac function after myocardial infarction. However, there is, as yet, little evidence with which to assess the long-term clinical effects of this treatment [4]. Although most studies so far reported have used BMSCs for therapeutic angiogenesis, those in which G-CSF-mobilized PBSCs were used gave comparable, mild improvements in cardiovascular lesions [5], and both sources are generally regarded as adequate for therapeutic angiogenesis, just as they are for hematopoiesis. To date, some studies have shown that EPCs, as well as HPCs, are demonstrably mobilized by G-CSF [6-8]. However, this depends on how EPCs are defined and interpreted: we can measure increases or decreases in EPC numbers following G-CSF administration, depending on how EPCs are defined [9].

The current characterizations of EPCs have been based on phenotype and on colony assays. HPCs are routinely defined for clinical use by their expression of CD34 or CD133 [10]. A link between CD34/CD133 expression and the EPC phenotype was proposed almost from the initial discovery of circulating EPCs [11,12], but recent studies have indicated that cells expressing CD133 and their progeny remain hematopoietic, and only CD34+CD133- cells are true EPCs [13,14]. True EPCs are defined as cells that, in culture over 3–4 weeks on collagen, can give rise to endothelial outgrowth cells (EOCs) [14-16], whereas cells that give rise over 5–6 days to colonies on fibronectin (colony-forming unit endothelial progenitor cells) (CFU-EPCs), formerly proposed to be EPCs [17], are now recognized to be generated by monocytes [16,18,19]. CFU-EPCs stain for many endothelial markers [20,21] but also retain CD14 expression [22]. Monocytes can themselves mimic endothelial cells (ECs) by upregulating expression of many markers held to be endothelial, and have probably been mistaken for ECs in many investigations [22,23]. Although it is implicit in many studies that the observed clinical benefit is delivered by EPCs, which are ultimately incorporated as ECs into new vasculature, it is becoming apparent that neovascularization can also be promoted indirectly by cells that release paracrine factors that promote angiogenesis without being incorporated as ECs [24]. Although such cells may not be true EPCs, their proangiogenic effect may be important, and this may be why so many different cell phenotypes have been proposed to be EPCs [25,26].

To date, G-CSF PBSC mobilization has been generally regarded as a practical and feasible source of cells for therapeutic angiogenesis [27-29]. However, a recent meta-analysis reported that G-CSF infusion alone had no significant clinical benefit in myocardial infarction [30], and it was reported that G-CSF-mobilized PBSCs were less effective in inducing ulcer healing than were BMSCs [31]. The mechanism by which G-CSF may mobilize EPCs and/or enhance angiogenesis is still unknown. Similarly, the effects of G-CSF on ECs and the vasculature have not been extensively studied [32]. In this study, we have investigated circulating EPC activity following HPC mobilization by G-CSF administration, and the direct effects of G-CSF on *in vitro* and *in vivo* correlates of angiogenesis.

## Materials and methods

### Animals

Male C57B6J mice aged 10–12 weeks were purchased from Charles River Laboratories (Tranent, UK) or Harlan Olac Ltd (Loughborough, UK). Experimental procedures were approved by the University of Edinburgh ethics committee, and were authorized by the Home Office under the Animals (Scientific Procedures) Act 1986.

### Cell sources and sampling

Peripheral venous blood samples from healthy adults (normal PB) were collected into heparin and from cord blood (CB) following elective caesarean section. For sequential studies, healthy PBSC donors (mobilized PB donors) and PBSC transplant patients (mobilized PB patients) donated 10 mL of venous PB before G-CSF mobilization (pre-G-CSF); at apheresis harvest (post-G-CSF), and 1–2 months after harvest (follow-up). The G-CSF protocol used in this work was the standard local clinical mobilization regimen. The G-CSF (lenograstim) dose for healthy donors was  $10 \mu\text{g kg}^{-1} \text{d}^{-1}$ , given for four consecutive days before collection of post-G-CSF cells (at apheresis) on day 5. Patient samples were subjected to chemotherapy — salvage chemotherapy (lymphoma patients) or cyclophosphamide (multiple myeloma patients) — followed by G-CSF (lenograstim), starting at least 24 h following the last dose of chemotherapy —  $5 \mu\text{g kg}^{-1} \text{d}^{-1}$  (lymphoma patients) or  $10 \mu\text{g kg}^{-1} \text{d}^{-1}$  (multiple myeloma patients) — and given for 6–7 days before collection of post-G-CSF cells when CD34+ counts exceeded  $10 \times 10^6 \text{L}^{-1}$ . Pre-G-CSF treatment cells were collected 10–30 days prior to G-CSF administration. Healthy adult donors (for allogeneic transplant) are the primary study subjects, and results for patients are included for comparison. Further clinical, hematologic and laboratory data are reviewed elsewhere (J. Crawford, MD thesis, University of Edinburgh, submitted). Appropriate ethical informed consent was obtained from subjects in all cases. Mononuclear cells (MNCs) were isolated by buoyant density centrifugation over Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden).

### Isolation of short-term (2 h) plastic-adherent MNCs

MNCs ( $30 \times 10^6$ ) in 5 mL of IMDM (Invitrogen, Paisley, UK) containing 10% fetal bovine serum (Sigma, Dorset, UK) were plated in  $25\text{-cm}^2$  Corning tissue culture flasks (Fisher Scientific, Loughborough, UK) and incubated at  $37^\circ\text{C}$ . After 2 h, adherent cells were detached using 1 mL of trypsin–EDTA in saline (Sigma). Harvested cells were resuspended in IMDM and characterized by flow cytometry for use in further experiments.

### Flow cytometry analysis and sorting

Cells were directly stained and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK), using CELLQUEST PRO software for phenotypic expression of surface

markers, and analyzed using FCS EXPRESS (DeNovo Software, Los Angeles, CA, USA), as described previously [9]. Cells were sorted using a FACS Aria flow cytometer (Becton Dickinson), using DIVA software. Sorted populations were recovered and characterized by further analysis. The anti-human monoclonal antibodies used for flow cytometry included anti-CD34–fluorescein isothiocyanate, anti-CD14–phycoerythrin, anti-CD45–PerCP (Becton Dickinson), and anti-CD133–allophycocyanin (Myltenyi Biotec, Bisley, UK).

### CFU-EPCs

This assay, based on the method of Hill *et al.* [17], was performed using a commercial kit according to the manufacturer's recommendations (Stem Cell Technologies, Grenoble, France). As previously described [9,20,21],  $5 \times 10^6$  unmodified MNCs were resuspended in EndoCult Liquid Medium (Stem Cell Technologies) and plated on fibronectin-coated six-well plates (Becton Dickinson) for 2 days. The non-adherent cells were recovered, resuspended in fresh medium, and transferred to a fibronectin-coated 24-well plate (Becton Dickinson) at  $1 \times 10^6$  per well in the presence or absence of G-CSF ( $100 \text{ ng mL}^{-1}$ ) for a further 3 days; the colonies per well were then counted, and the EPC frequency was calculated. The concentration of G-CSF used in this and other *in vitro* assays described below was based on a previously determined optimal dose for CD34+ PBSC expansion/differentiation to neutrophils [33].

### Culture of EOCs

EOC culture was performed as described by Ingram *et al.* [34]. Briefly,  $30 \times 10^6$  MNCs from normal PB or  $10 \times 10^6$  MNCs from CB were resuspended in endothelial growth medium (EBM-2; Lonza, Slough, UK) and plated onto type 1 rat tail collagen-coated six-well tissue culture plates (Becton Dickinson). The cells were incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for 3–4 weeks. The medium was changed every 2 days for 7 days, and then twice a week until first passage. Colonies were counted when they became evident but before they became confluent.

### *In vitro* vascular tubule formation assay

Matrigel matrix (Becton Dickinson) solution was thawed overnight at  $4^\circ\text{C}$ , and all plasticware was precooled. Human umbilical vein endothelial cells (HUVECs) (Lonza) and EOCs were resuspended at  $1 \times 10^5 \text{ mL}^{-1}$  in EBM-2 in the presence or absence of G-CSF ( $100 \text{ ng mL}^{-1}$ ). Five hundred microliters of cells were added to duplicate wells precoated with  $250 \mu\text{L}$  of Matrigel that had been allowed to solidify for 1 h at  $37^\circ\text{C}$ . Capillary structures and EC networks were examined by phase contrast microscopy ( $\times 40$  lens), using an inverted microscope (Nikon Eclipse TS100-F, Nikon Instruments, Kingston Upon Thames, UK). Pictures were taken at 4 h and at 22 h. The EC network was quantified from the image fields by scoring the number of cell–cell connections.

### *In vitro* angiogenesis: aortic ring assay

C57Bl6 mice were killed by asphyxiation in  $\text{CO}_2$ . The thoracic aorta was removed, washed in serum-free MCDB 131 medium (Invitrogen), cleaned of periadventitial tissue, and divided into 1-mm rings. Aortic rings were embedded in  $200 \mu\text{L}$  of Matrigel (Becton Dickinson) and incubated at  $37^\circ\text{C}$  in serum-free MCDB 131, with heparin, ascorbic acid and GA1000 (Cambrex Biosciences, Wokingham, UK) in the presence or absence of G-CSF ( $100 \text{ ng mL}^{-1}$ ). The medium was changed every 48 h. All assays were performed in triplicate. The growth of new vessels was counted at day 4 and day 8 by light microscopy.

### Subcutaneous sponge implantation assay for *in vivo* vascularization

Mice were anesthetized with halothane, and a sterilized sponge cylinder (0.5 cm diameter, 1 cm long) (Caligen Foam, Accrington, UK) was implanted subcutaneously on each flank. Each animal had an intervention-impregnated sponge [growth-factor-reduced (GFR)-Matrigel + G-CSF] on one side and a control, vehicle-impregnated sponge (GFR-Matrigel alone) on the other side. Twenty days after implantation, mice were killed, and sponges were excised. Sponges were fixed in 4% formalin and embedded in paraffin wax. Sections (5  $\mu$ m) were stained with hematoxylin–eosin for identification of blood vessels, as previously described [35]. Vessel density within sponges was determined using the mean of triplicate Chalkley counts on each of two sections per sponge [36].

### Statistical analysis

Unless otherwise stated, continuous variables are reported as mean  $\pm$  standard error of the mean. Statistical analyses were performed with GRAPH PAD PRISM 4 (Graph Pad Software, La Jolla, CA, USA), using two-tailed Student's *t*-test, Mann–Whitney *U*-test or Wilcoxon paired tests where appropriate. A *P*-value of  $< 0.05$  was considered to indicate statistical significance.

### Results

#### The effects of *in vivo* administration of a PBSC-mobilizing G-CSF regimen on indicators of angiogenesis

**The induced depression of CFU-EPC capacity in PB MNCs following G-CSF administration is profound but transient**—When individual healthy subjects (PBSC donors) were followed sequentially, G-CSF administration caused a profound decline in CFU-EPC activity from normal levels, which recovered with time (Fig. 1A). The CFU-EPC activity was virtually abolished after G-CSF administration as compared with the pre-G-CSF sample ( $P < 0.001$ , paired *t*-test,  $n = 21$ ). CFU-EPC activity returned to almost basal levels in follow-up samples after 1–2 months following completion of G-CSF treatment ( $P < 0.01$ , paired *t*-test,  $n = 13$ ). Similar depression and recovery of CFU-EPC activity following G-CSF mobilization of PBSCs (for autologous transplantation) was seen in a large series of hematology patients (data not shown).

**G-CSF-mobilized PB MNCs are unable to generate CFU-EPCs and these are not recovered by monocyte enrichment**—Confirming what we have previously reported [9], CFU-EPCs were most prevalent in normal PB MNCs and were virtually absent from G-CSF-mobilized PB MNCs, whether from patient or healthy donor sources (Fig. 1B). We found that CFU-EPCs were slightly increased (e.g. from 21 to 25 per  $10^6$  MNCs,  $n = 6$ ) when normal PB MNCs were enriched for monocytes (to around 80% CD14+) by 2 h of adherence on uncoated culture plates. Further enrichment of monocytes (to 98%) by CD14+ selection by FACS further increased CFU-EPCs (e.g. from 25 to 41 per  $10^6$  MNCs,  $n = 6$ ) and completely removed CFU-EPCs from the CD14-depleted adherent cells. CFU-EPCs were not found in CD34-enriched ( $> 90\%$ ) or CD133-enriched ( $> 90\%$ ) MNCs from different HPC-rich sources (Table 1).

In a larger series studied by plastic adherence enrichment alone, normal PB MNCs generated a mean of 37 CFU-EPCs per  $10^6$  cells plated, CB MNCs generated fewer than five CFU-EPCs per  $10^6$  cells plated, and G-CSF-mobilized PB MNCs were virtually unable to form any CFU-EPCs (0.6 CFU-EPCs per  $10^6$  cells plated (Fig. 1C). Whereas enrichment of CD14+ cells by plastic adherence increased the number of CFU-EPCs slightly in normal PB MNCs and significantly in CB MNCs, no CFU-EPCs were seen when CD14-enriched cells from G-CSF-mobilized MNCs were cultured.



**Mobilization of PB HPCs with G-CSF alters the phenotype proportions of CD34+ cell subpopulations**—G-CSF-mobilized PB samples had a more than 10-fold higher proportion of CD34+ cells than normal PB samples (Fig. 2A). In agreement with our earlier studies [9], G-CSF-mobilized PB samples had markedly higher coexpression of CD133 by CD34+ cells ( $81.4\% \pm 10.5\%$ ) than normal PB samples ( $23.1\% \pm 18.2\%$ ). This was true for both healthy donors ( $P < 0.001$ , Mann-Whitney *U*-test,  $n = 9$ ) and for autologous patients (Fig. 2B). Furthermore, in contrast to normal PB, in which a mean of 37% of CD34+ cells were CD45<sup>low</sup>, G-CSF-mobilized PB samples contained very low proportions of CD34+CD45<sup>low</sup> cells (mean G-CSF-mobilized PB autologous, 1.64%; mean G-CSF-mobilized PB allogeneic, 1.63%) ( $P < 0.001$ , Mann-Whitney *U*-test,  $n = 9$ ) (Fig. 2C), and their absolute numbers were little increased in G-CSF-mobilized PB as compared with normal PB, in contrast to total CD34+ numbers (Fig. 2D).

**G-CSF-mobilized PB MNCs are unable to generate EOCs**—CB MNCs ( $n = 15$ ) reliably generated EOC colonies when  $10 \times 10^6$  MNCs were plated. For normal PB MNCs ( $n = 12$ ), at least  $30 \times 10^6$  MNCs had to be plated. G-CSF-mobilized blood MNCs were unable to form any EOC colonies for up to  $30 \times 10^6$  MNCs plated ( $n = 7$ ), either for healthy donors or for patients (Table 2).

**EOC potential is associated with CD34+CD133– cells**—Enrichment of the CD34+ fraction of CB MNCs by magnetic bead cell sorting ( $> 90\%$  purity) showed that this fraction was the source of all EOC colonies. No colonies were found in CD34-depleted MNCs (Table 2). Conversely, the CD133-enriched fraction from CB MNCs ( $> 90\%$  purity) gave no EOC colonies, and all of the EOC colonies were generated from the CD133-depleted fraction. When the CD133– fraction was further sorted into CD34+ and CD34– fractions, only the CD34+ (CD133–) fraction gave EOC colonies. The number of cells required to produce EOC colonies fell dramatically with enrichment of CD34+CD133– cells. Enrichment for the CD34+CD133– cell population increased the frequency of EOC generation from CB MNCs, but not when G-CSF-mobilized blood MNCs were used.

### The effect of direct addition of exogenous G-CSF on *in vitro* indicators of angiogenesis

#### The number of CFU-EPCs is reduced by addition of G-CSF to colony cultures

—The addition of G-CSF to normal PB MNCs *in vitro* significantly reduced the CFU-EPC frequency as compared with controls without G-CSF ( $P < 0.01$ , paired *t*-test,  $n = 8$ ) (Fig. 3). The addition of  $100 \text{ ng mL}^{-1}$  vascular endothelial growth factor (VEGF) or stromal cell-derived factor-1 (SDF-1) to the wells to which G-CSF had been added did not rescue colony formation (data not shown).

**Tubule formation by HUVECs and EOCs is reduced by G-CSF**—Vascular tubule formation by HUVECs in Matrigel showed a significant reduction at 22 h in the presence of G-CSF as compared with controls ( $P < 0.01$ , paired *t*-test,  $n = 5$ ) (Fig. 4C); a paired example is shown in Fig. 4A,B. The addition of  $100 \text{ ng mL}^{-1}$  VEGF or SDF-1 to the wells with G-CSF did not rescue tube formation (data not shown). EOCs grown on collagen behave like HUVECs in many ways, and form tubules in Matrigel. As with HUVECs, tubule formation by EOCs in Matrigel was inhibited by G-CSF ( $n = 5$ ) (Fig. 4D–F).

**Angiogenesis from mouse aortic rings *in vitro* is reduced by G-CSF**—The numbers of vessels formed from murine aortic rings cultured *in vitro* and scored after 4 and 8 days were reduced in the presence of G-CSF as compared with controls at both time points ( $P < 0.05$ , paired *t*-test,  $n = 4$ , means of triplicates). Results scored at 8 days are shown in Fig. 5C. A paired example is shown in Fig. 5A,B.

***In vivo* spontaneous angiogenesis in subcutaneous sponge implants in mice is inhibited by G-CSF**—Control vehicle-impregnated sponges (GFR-Matrigel only) and G-CSF-impregnated sponges (G-CSF in GRF-Matrigel) excised after 20 days following implantation both appeared red on gross inspection, with lace-like coverings of blood vessels. They also both showed infiltration of organized matrix and an abundance of blood vessels. On histologic examination, all sponges exhibited vascularization, but G-CSF-impregnated sponges had significantly fewer blood vessels than controls when scored by Chalkley counts ( $P < 0.001$ , Mann–Whitney  $U$ -test,  $n = 4$ ) (Fig. 6). A group of mice ( $n = 4$ ) implanted with untreated sponges (no GFR-Matrigel vehicle) on both flanks exhibited a similar level of vasculogenesis as that seen in vehicle-impregnated sponges (GFR-Matrigel), demonstrating that GFR-Matrigel as vehicle has no intrinsic effect on vascularization (not shown).

## Discussion

It is well established that G-CSF administration successfully mobilizes progenitor cells to PB, and these cells are able to reconstitute the hematopoietic system; therefore, it is thought that G-CSF mobilization might also increase the number of circulating EPCs. In this study, we used PBSCs from subjects receiving G-CSF for HPC mobilization, to examine the possibility that EPCs are mobilized into the circulation concurrently. We were unable to detect EPCs in G-CSF-mobilized PBSCs, using several of the published EPC phenotypes. G-CSF administration for HPC mobilization not only failed to mobilize EPCs, but also inhibited angiogenesis *in vitro* and *in vivo*.

### Neither G-CSF-mobilized PB MNCs nor enriched monocytes are able to generate CFU-EPCs

Initial observations showed that, following administration of G-CSF to healthy allogeneic PBSC donors, there was a profound depression of CFU-EPC generation [9]. This reduction in endothelial colony potential has been consistently shown by independent observers in our group and in over 70 different samples studied. Longitudinal analysis of sequential PB samples from healthy donors undergoing PBSC mobilization showed that CFU-EPC numbers were severely reduced immediately after G-CSF administration, but returned to almost pretreatment levels within 2 months (Fig. 1A). Similar results have been obtained in hematologic malignancy patients undergoing PBSC mobilization for autologous transplantation, and may indicate that preceding chemotherapy does not markedly affect many putative laboratory measures of PBSC or endothelial function. Although CFU-EPCs were originally proposed by Hill et al. [17] as a correlate of EPC frequency, it is now established that they represent an expression of the activity of CD14<sup>+</sup> monocytes [37], which possibly constitute a key proangiogenic monocyte subpopulation, not related to any HPC population. Monocyte enrichment by plastic adherence increased the frequency of CFU-EPC generation from umbilical cord MNCs but did not increase the frequency of CFU-EPC generation from G-CSF-mobilized blood MNCs (Fig. 1C), which, in contrast to CB monocytes, appear to be unresponsive in this assay.

A recognized, strong, inverse correlation between CFU-EPC frequency and cardiovascular risk has been reported extensively (reviewed in [20,21]). This accumulation of reports is not trivial, and indicates that CFU-EPC measurement can assess some aspects of angiogenic capacity. Thus, although CFU-EPC measurement seems not to be an indicator of EPC frequency, as originally proposed, depressed CFU-EPC activity in G-CSF-mobilized samples probably reflects reduced monocyte proangiogenic capacity.



### G-CSF-mobilized PB MNCs are unable to generate EOCs

There are a number of claims that G-CSF mobilizes EPCs [6-8], but these depend on how EPCs are defined and interpreted. A very few studies are based on a reported increase in CFU-EPC frequency [7], and nearly all are based on putative phenotype characterization of EPCs. Although the proposed phenotypes for EPC have been dominated by variants based on coexpression of CD34 and CD133, the definitive phenotype of an EPC remains elusive. Recently, it has been reported that true circulating EPCs (EOCs) are CD34-positive but CD133-negative and CD45-negative, whereas cells expressing CD133 and CD45 remain hematopoietic and do not give rise to true ECs [13,14,38]. Previous observations showed that the HPCs in G-CSF-mobilized blood are predominantly CD34+CD133+ [9,39], so by phenotype alone there is no evidence of mobilization of EOCs (contained in the CD34+CD133- subpopulation) by G-CSF (Fig. 2B), and nor is there evidence that CD34+ cells with low or negligible expression of the panleukocyte marker CD45 are selectively mobilized by G-CSF (Fig. 2C). Indeed, no EOCs could be cultured from G-CSF-mobilized blood MNCs ( $30 \times 10^6$ ) from either autologous patients or allogeneic donors, whereas, in most cases, at least one EOC colony can be found in comparable normal PB MNCs, so there is no evidence that EOCs are mobilized by G-CSF. As there is little or no selective mobilization of CD34+CD133-CD45- cells by G-CSF, this may account for the failure to find EOCs in these samples. Furthermore, enrichment for the proposed EOC precursor population (CD34+CD133-) by magnetic beads increased the frequency of EOC generation from umbilical cord MNCs, but not from G-CSF-mobilized blood MNCs. We have no evidence to judge whether EOCs are present at low frequency/absent or inactive in G-CSF-mobilized MNCs.

### G-CSF has a direct inhibitory effect on angiogenesis

Although some of the effects of G-CSF on CFU-EPCs *in vivo* might result from alteration of the balance of different cell types in the circulation and dilution of some MNC subpopulations by others, it can be shown that G-CSF has a direct effect *in vitro* on CFU-EPCs. In paired MNC samples, the addition of G-CSF resulted in a decrease in CFU-EPC frequency (Fig. 3). Furthermore, the addition of known angiogenesis-promoting cytokines such as VEGF or SDF-1 to the G-CSF-treated MNCs did not rescue colony formation. Similarly, it can be shown that G-CSF depresses the expression *in vitro* of accepted endothelial cell functions, such as the formation of cell-cell links in human EC (HUVEC and EOC) tubule formation in Matrigel (Fig. 4) and in microvessel outgrowth from mouse aortic rings (Fig. 5).

The direct effect of G-CSF on angiogenesis was ultimately confirmed with the use of an *in vivo* mouse model of angiogenesis (subcutaneous sponge implantation). Localized G-CSF substantially inhibited spontaneous vascularization of sponges *in vivo* (Fig. 6), in direct contrast to what was seen in paired sponges lacking G-CSF in the same animals. Honold *et al.* [8] showed that EPCs in G-CSF-mobilized samples were transiently dysfunctional, owing to the cleavage of the chemokine receptor CXCR4, which is directly involved in stem cell homing. Thus, the observed reduction of endogenous blood vessel formation in the G-CSF-treated sponge may reflect a localized decline in the ability to recruit potential murine angiogenic cells. Preliminary evidence from our current work suggests that G-CSF may downregulate the expression of certain cell surface receptors and adherence molecules, which may impair the ability of cells to function in certain environments; this could explain the observed CFU-EPC depression and might be important in endothelial function and/or angiogenesis. This is currently under investigation.

In agreement with our findings, a recent meta-analysis reported that G-CSF infusion alone has no significant clinical benefit in myocardial infarction [30], and G-CSF-mobilized

PBSCs were reported to be less effective in inducing ulcer healing than BMSCs [31]. However, a significant number of reports to date have shown that cellular therapies employing G-CSF-mobilized cells have some clinical benefit [27-29]. Thus, although G-CSF may not selectively mobilize true EPCs as defined by EOCs, and although it may inhibit monocyte proangiogenic activity and EC angiogenic activity, G-CSF does induce an increase in the number of circulating HPCs, which might home to ischemic lesions [27] and could therefore provide a paracrine effect without any incorporation into new vessels. These may be equivalent to the cells provided from bone marrow, and if that is the principal effect required in some aspects of therapeutic vascularization, then mobilized PBSCs may be as beneficial as BMSCs in clinical use.

In summary, this study has shown that there is a profound reduction in the number of CFU-EPCs following G-CSF administration, which recovers with time. To the best of our knowledge, we are the first to show that there is no evidence of circulating EOCs following G-CSF administration for mobilization of HPCs. G-CSF-mobilized PBSC were predominantly CD34+CD133+ cells, which are almost certainly hematopoietic cells. The presence *in vitro* of exogenous G-CSF had a direct antiangiogenic effect that was not abrogated by the addition of proangiogenic factors.

## Acknowledgments

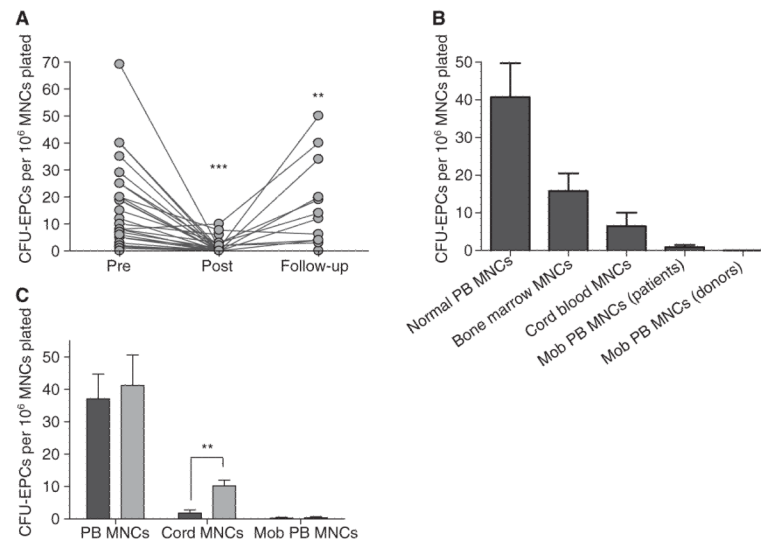
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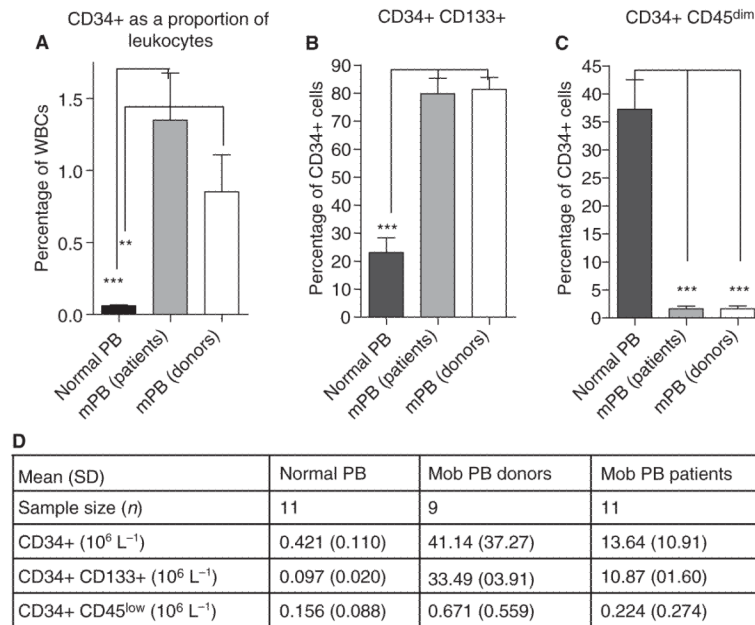
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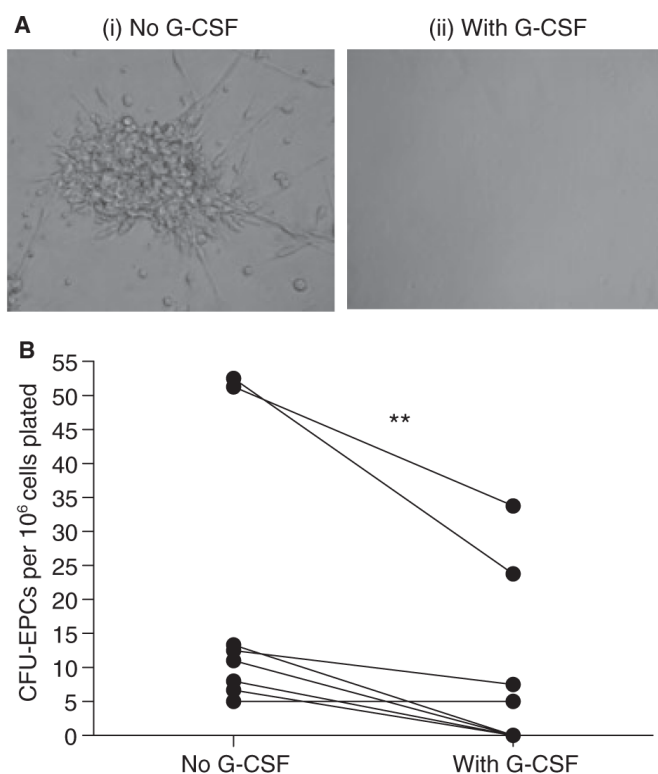
**Fig. 1.**

Influence of administration of granulocyte colony-stimulating factor (G-CSF) for hematopoietic progenitor cell (HPC) mobilization (Mob) on colony-forming unit endothelial progenitor cells (CFU-EPCs) in peripheral blood (PB) mononuclear cells (MNCs). (A) There was a fall in CFU-EPCs following G-CSF administration to healthy adult HPC donors and a subsequent rise in CFU-EPCs at 1–2-month follow-up after mobilization. (B) CFU-EPCs in normal PB MNCs and in HPC-rich MNC sources. (C) CFU-EPCs in MNCs (black) and following monocyte (CD14) enrichment by plastic adherence (grey). A  $P$ -value of  $< 0.05$  was considered to indicate statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Pre, pre-G-CSF administration; Post, post-G-CSF administration.

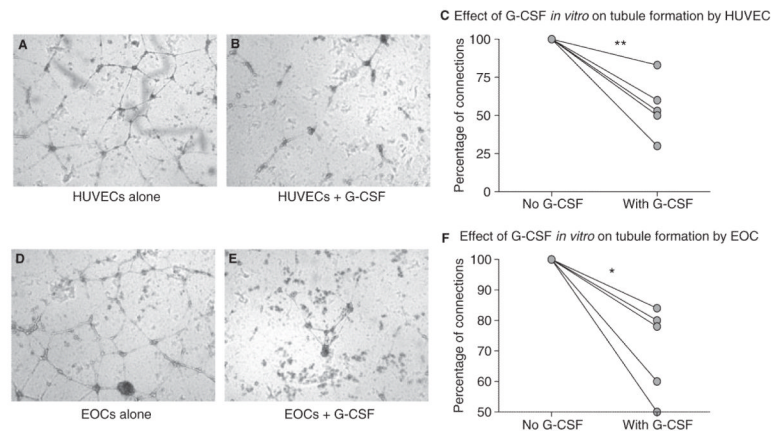


**Fig. 2.**

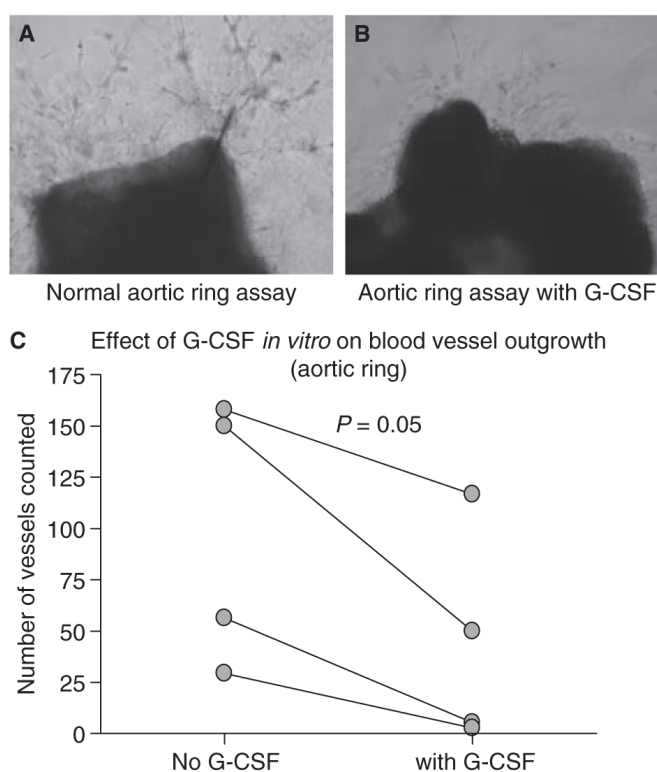
Influence of administration of granulocyte colony-stimulating factor (G-CSF) on hematopoietic progenitor cell (HPC) mobilization on CD34+ cells and CD34+ subpopulations in peripheral blood (PB) leukocytes. (A) CD34+ cells as a proportion of total leukocytes in normal PB and in G-CSF-mobilized PB from healthy donors and from hematologic malignancy patients in remission (mPB). (B) The proportion of CD34+ cells coexpressing CD133 in normal PB and in G-CSF-mobilized PB from healthy donors and from hematologic malignancy patients in remission. (C) The proportion of CD34+ cells with low to negligible CD45 expression in normal PB and in G-CSF-mobilized PB from healthy donors and from hematologic malignancy patients in remission. (D) Absolute numbers of circulating HPC subpopulations per liter of PB: all CD34+ cells; CD34+ cells coexpressing CD133; and CD34+ cells low in CD45 expression. The results are calculated from research laboratory determination of total leukocyte counts and proportional subpopulations, expressed as mean  $\pm$  standard deviation (SD). A *P*-value of  $< 0.05$  was considered to indicate statistical significance (\**P*  $< 0.05$ ; \*\**P*  $< 0.01$ ; \*\*\**P*  $< 0.001$ ). Mob, mobilized; WBC, white blood cell.



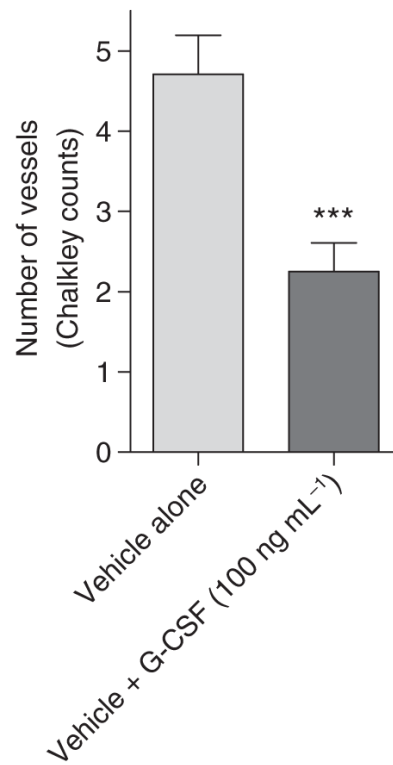
**Fig. 3.** Influence of exogenous granulocyte colony-stimulating factor (G-CSF) *in vitro* on colony-forming unit endothelial progenitor cells (CFU-EPCs). (A) Representative microscopy images of CFU-EPC formation (i) by normal peripheral blood mononuclear cells (MNCs) and (ii) in the presence of exogenous G-CSF (100 ng mL<sup>-1</sup>). There was a significant reduction in MNC CFU-EPCs in the presence of G-CSF as compared with paired samples without G-CSF. A *P*-value of < 0.05 was considered to indicate statistical significance (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001).

**Fig. 4.**

Influence of exogenous granulocyte colony-stimulating factor (G-CSF) *in vitro* on tubule formation. (A, B) Representative microscopy images of (A) normal tubule formation by human umbilical vein endothelial cells (HUVECs) and (B) tubule formation by HUVECs in a paired culture with exogenous G-CSF added. (C) There was a significant reduction in HUVEC tubule connections in the presence of G-CSF as compared with paired samples without G-CSF. (D, E) Representative microscopy images of (D) normal tubule formation by endothelial outgrowth cells (EOCs) and (E) tubule formation by EOCs in a paired culture with exogenous G-CSF added. (F) There was a significant reduction in EOC tubule connections in the presence of G-CSF as compared to paired samples without G-CSF. A *P*-value of  $< 0.05$  was considered to indicate statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).



**Fig. 5.** Influence of exogenous granulocyte colony-stimulating factor (G-CSF) *in vitro* on microvessel outgrowth from mouse aortic rings. Representative microscopy images of (A) normal microvessel outgrowth from mouse aortic ring and (B) microvessel outgrowth from a paired aortic ring sample from the same mouse in the presence of added exogenous G-CSF. (C) There was a significant reduction in microvessel outgrowth from mouse aortic rings in the presence of G-CSF as compared with paired samples without G-CSF.



**Fig. 6.** Inhibition of angiogenesis *in vivo* by granulocyte colony-stimulating factor (G-CSF). There was a reduction of spontaneous vascularization of subcutaneously implanted sponges containing G-CSF in growth-factor-reduced Matrigel as compared with contralateral sponges in the same animal containing growth-factor-reduced Matrigel alone. A *P*-value of  $< 0.05$  was considered to indicate statistical significance (\**P*  $< 0.05$ ; \*\**P*  $< 0.01$ ; \*\*\**P*  $< 0.001$ ).



**Table 1**

Effect of subpopulation enrichment/depletion on colony-forming unit endothelial progenitor cells (CFU-EPCs) in mononuclear cell (MNC) populations

Source	Number of CFU-EPCs per 10 <sup>6</sup> MNCs plated
Unfractionated MNCs *	21
2-h adherent cells (> 80% CD14+) *	25
2-h non-adherent cells *	2
CD14-enriched (> 98%) *	41
CD14-depleted *	0
CD34-enriched (> 90%) †	0
CD34-depleted †	3
CD133-enriched (> 90%) †	0
CD133-depleted †	8

CFU-EPCs are slightly increased when normal peripheral blood MNCs are enriched for monocytes (to about 80% CD14+) by 2 h of adherence on uncoated culture plates. Further enrichment of monocytes (to 98%) by CD14+ selection by fluorescence-activated cell sorting further increased CFU-EPCs and completely removed CFU-EPCs from the CD14-depleted plastic-adherent cells. CFU-EPCs were not found in CD34-enriched (> 90%) or CD133-enriched (> 90%) MNCs from different HPC-rich cell sources. Their depleted column eluates tend to show reduced CFU-EPC activity as compared with the unfractionated starting MNCs, which may imply some loss of CFU-EPC activity by retention on columns by adhesion, implying these cells are adherent.

\* Paired normal peripheral blood samples ( $n = 6$ ).

† Unpaired HPC-rich samples (bone marrow, cord blood, mobilized blood).

**Table 2**

Attainment of endothelial outgrowth cell (EOC) colonies from different mononuclear cell (MNC) sources and subpopulations

Source (cord blood MNCs)	EOC colonies found	Number of MNCs plated for one EOC colony
Unfractionated MNCs	Yes	$3 \times 10^6$
CD133-enriched (> 90%)	No	(> $1.3 \times 10^6$ )
CD133-depleted	Yes	$0.5 \times 10^6$
CD34-enriched (> 90%)	Yes	$1.7 \times 10^6$
CD34-depleted	No	(> $3 \times 10^6$ )
CD34+CD133+ (> 90%)	No	(> $1.65 \times 10^6$ )
CD34+CD133-depleted	Yes	$0.002 \times 10^6$

EOCs can be routinely cultured from cord blood MNCs plated at  $5\text{--}10 \times 10^6$  per well on collagen. For normal adult peripheral blood MNCs,  $30 \times 10^6$  MNCs per well are required to give approximately one EOC colony. From cord blood MNCs separated by magnetic beads, CD34-enriched cells form EOCs, but their CD34-depleted eluates do not. CD133-enriched cells do not form EOCs, but their CD133-depleted eluates do: if the CD133-depleted cells are further fractionated according to CD34 expression, the CD34-enriched (CD34+CD133–) cells form EOCs but the CD34– eluates do not.